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Urolithins Display both Antioxidant and Pro-oxidant Activities Depending on Assay System and Conditions

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Supporting Information

ABSTRACT: The biological effects of polyphenolic ellagitannins are mediated by their intestinal metabolites, urolithins. This study investigated redox properties of urolithins A and B using ORAC assay, three cell-based assays, copper-initiated pro-oxidant activity (CIPA) assay, and cyclic voltammetry. Urolithins were strong antioxidants in the ORAC assay, but mostly pro-oxidants in cell-based assays, although urolithin A was an antioxidant in cell culture medium. Parent compound ellagic acid was a strong extracellular antioxidant, but showed no response in the intracellular assay. The CIPA assay confirmed the pro-oxidant activity of ellagitannin metabolites. In the cell proliferation assay, urolithins but not ellagic acid decreased growth and metabolism of HepG2 liver cells. In cyclic voltammetry, the oxidation of urolithin A was partly reversible, but that of urolithin B was irreversible. These results illustrate how strongly measured redox properties depend on the employed assay system and conditions and emphasize the importance of studying pro-oxidant and antioxidant activities in parallel.

KEYWORDS: *antioxidant, cytotoxic, ellagic acid, phenolics, pro-oxidant, urolithin*

INTRODUCTION

Urolithins are intestinal metabolites of polyphenolic ellagitannins, and they consist of a benzopyranone ring structure with different phenolic hydroxylation patterns (Figure 1A).[1,2]

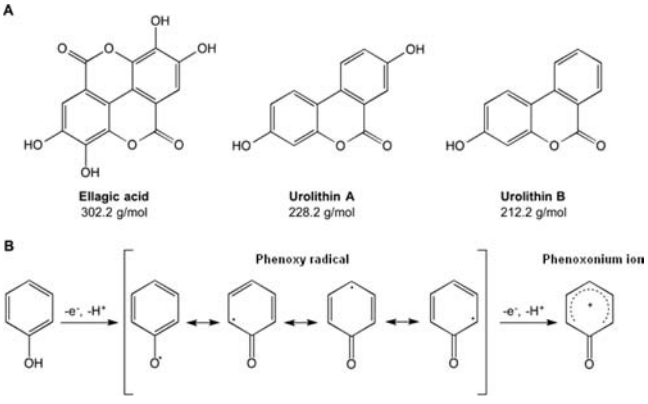

Figure 1. Molecular structures of ellagitannin metabolites (A) and reaction scheme for the oxidation of phenol to phenoxy radical and phenoxonium ion (B).

Urolithin A, isourolithin A, and urolithin B are the most common urolithins found in humans and animals.[3−6] They are produced by the action of unidentified colonic bacteria after ellagitannins are hydrolyzed to ellagic acid in the acidic environment of the stomach. Because both ellagitannins and ellagic acid are extremely poorly absorbed,[5] urolithins appear to be responsible for biological activities related to the intake of ellagitannins. Ellagitannins are strong natural antioxidants, and there is a growing body of evidence on their beneficial health effects, including prevention of cardiovascular diseases and cancers.[7−9] The antioxidant capacity is thought to be central for the health effects of polyphenols, but they may also exert in vivo effects by other mechanisms such as binding to enzymes and receptors.[10] The richest food sources of ellagitannins are pomegranate, walnuts, and berries of the genera *Rubus* (e.g., blackberry, cloudberry, and raspberry) and *Fragaria* (strawberry).[8,11]

In recent years, the dual redox nature of plant-derived polyphenols has become widely acknowledged, emphasizing the need to investigate both the antioxidant and pro-oxidant capacities of the compounds because their physiological effects may depend upon their behavior as either an antioxidant or a pro-oxidant.[12] Urolithins have been reported to possess antioxidant and anti-inflammatory properties; however, there have been contradictory reports on their antioxidant capacity, and their pro-oxidant properties have not been studied at all.[1,13−20] Interestingly, only one chemical in vitro assay, the oxygen radical absorbance capacity (ORAC) assay, has identified urolithins as antioxidants.[14,15] The ORAC assay measures antioxidant inhibition of peroxyl radical induced oxidation and thus reflects classical radical chain-breaking antioxidant activity by hydrogen atom transfer (HAT) mechanism.[21,22] Of the other assays employed to study urolithins, DPPH, FRAP, and $ABTS^{\bullet+}$ assays[1,15,18] are based on the single electron transfer (SET) mechanism, whereas the xanthine/XOD and PMS/NADH systems[15,18] specifically measure the scavenging of superoxide anions. Unconjugated urolithins (Figure 1A) do not have other functional groups in addition to phenolic hydroxyl groups. The electron-withdrawing carboxyl group is part of a lactone ring and thus cannot promote the SET mechanism. All of this suggests that the antioxidant activity of urolithins is very likely mediated

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exclusively by the HAT mechanism. The hydrogen atom is donated by the phenolic hydroxyl group (Figure 1B).[23]

In this study, we aimed to characterize both antioxidant and pro-oxidant properties of urolithins A and B using chemical in vitro assays, cell-based assays, and electrochemistry. We were especially interested in evaluating the effect of urolithins on intracellular oxidation reactions and concomitantly prepared to critically assess existing cell-based antioxidant assays and to develop new assay modifications. In parallel with redox properties, we wanted to inspect how urolithins A and B affect proliferation of HepG2 cells.

MATERIALS AND METHODS

Chemicals. Resorcinol (purity > 99%), 2-bromobenzoic acid (for synthesis), chlorobenzene (>99%), dimethyl sulfoxide (DMSO, >99.9%), and potassium nitrate (p.a.) were purchased from Merck Schuchardt OHG (Hohenbrunn, Germany). 2,2′-Azobis[2-methyl-propionamidine] dihydrochloride (AAPH, 97%), copper(II)sulfate pentahydrate (>98%), 2′,7′-dichlorofluorescin diacetate (DCFH-DA, ≥97%), fluorescein (puriss. p.a.), and (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 2-Bromo-5-methoxybenzoic acid (>98%) was from Alfa Aesar GmbH (Karlsruhe, Germany) and ellagic acid (99.9%) from MP Biochemicals (Solon, OH, USA). Cell culture medium and supplements were from either Gibco (Invitrogen Corp., Paisley, Scotland, UK), Lonza (Verviers, Belgium), or Sigma-Aldrich. Qualified fetal bovine serum was from Gibco.

Synthesis of Urolithins. Urolithins A (3,8-dihydroxy-6*H*-dibenzo(*b*,*d*)pyran-6-one) and B (3-hydroxy-6*H*-dibenzo(*b*,*d*)pyran-6-one) were synthesized by the condensation of resorcinol with the appropriately substituted benzoic acids using protocols published earlier.[18,20] The identity and purity of the urolithins was confirmed by HPLC-DAD/MSD analysis.

Analysis of Urolithins and Ellagic Acid by HPLC-DAD/MSD. The HPLC analysis was performed using an Agilent 1100 instrument (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a binary pump delivery system, a degasser, an autosampler, a photodiode array detector (DAD), and a mass selective detector (MSD). The mobile phase consisted of eluents (A) 1% formic acid in water (v/v) and (B) acetonitrile with the following linear gradient: 20−40% B (0−5 min), 40−45% B (5−15 min), and 45−90% B (15−16 min) at a flow rate of 0.4 mL/min. The post run time was 6 min. The column (150 × 3.00 mm, 5 μm, 110 Å, C18, Gemini; Phenomenex) temperature was 25 °C and the sample injection volume, 10 μL. Urolithins and ellagic acid were monitored at 280 and 255 nm, respectively. The UV spectra were obtained by scanning from 190 to 600 nm. Full-scan mass spectra were recorded from *m*/*z* 100 to 1000 using atmospheric pressure ionization in a negative electrospray (API-ES) mode with nitrogen as a drying gas. Spray chamber parameters were as follows: drying gas flow, 12 L/min; gas drying temperature, 350 °C; nebulizer pressure, 35 psig; capillary voltage, 3000 V; fragmentor voltage, 200 V.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC of studied compounds was analyzed using the method of Huang et al.,[24] except that 80 μM EDTA was added to the phosphate buffer (pH 7.4) solution as described by Nkhili et al.[25] Analysis was performed with a microplate reader (Varioskan Flash, Thermo Scientific, Vantaa, Finland) equipped with a fluorescence detector and a dispenser. Stock solutions of urolithins A and B and ellagic acid (3.5 mM each) were prepared in DMSO. DMSO blank, different dilutions of studied compounds (2.5−10 μM), and Trolox standards (15−100 μM) were prepared in phosphate buffer (75 mM, pH 7.4) and pipetted (25 μL) to clear 96-well microplate wells (Greiner Bio-one, PS-microplate 96-well, U-shape). Fluorescein (81.6 nM) was added (150 μL) to the samples, and the plate was placed in the preheated (37 °C) microplate reader and incubated for 15 min before automatic dispension of 25 μL of AAPH (153 mM). After AAPH addition, the sample was mixed (5 s, 60 spm), and fluorescence was measured at 530 nm with excitation at 485 nm every minute for 80 min. The area under the oxidation curve (AUC) of each sample dilution was calculated, and the ORAC values of samples were then calculated by dividing the slope of blank corrected linear regression curve by that of the Trolox standard curve.

Cell Culture. The human hepatocellular carcinoma cell line HepG2 was cultured in Dulbecco's modified Eagle medium containing stabilized glutamine and supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, penicillin, and streptomycin in a 5% CO_2 atmosphere at 37 °C.

Cell-Based Antioxidant Activity Assays. For all three cellular antioxidant activity assay methods triturated HepG2 cells were seeded into 96-well plates at the density of 2×10^4 cells/100 μL/well. The plates with black walls and clear optic quality bottoms (Greiner Bio-one, CELLSTAR, 96-well, flat bottom) were used to optimize fluorescence detection. Cells were allowed to adhere and grow for 24 h before the antioxidant assay. All three assay methods deployed DCFH-DA as an oxidizable dye. The reduced DCFH-DA is nonfluorescent, but becomes fluorescent when it is oxidized in the medium to fluorescein diacetate or intracellularly to fluorescein after acetate groups have been removed by cellular esterases and DCFH has become trapped inside cells.[26] Therefore, DCFH-DA can be used to monitor oxidation in cell cultures (mainly extracellular oxidation in medium) or specifically intracellular oxidation. The antioxidant present in the assay decreases the increase of fluorescence. In our assays, the growth of fluorescence was monitored every 5 min for 75 min by a microplate reader using an excitation wavelength of 485 nm and an emission wavelength of 525 nm. All samples and controls were done in quadruplicate or sextuplicate. The stock solutions of studied compounds were dissolved in DMSO. The required dilutions of the stock solutions were made in 40% DMSO and diluted before assays 35-fold with complete culture medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to maintain the pH close to neutral (treatment medium). Two sets of control samples incubated with 1.14% DMSO but without antioxidant were prepared for all assays: the oxidant AAPH was not added to controls measuring unspecific fluorescence, whereas another set was prepared without cells. The latter control samples were used depending on the assay either to determine and subtract residual extracellular oxidation of fluorescence probe from the intracellular oxidation or to evaluate the effect of cells on the oxidation of dye in medium.

For measuring *antioxidant activity in cell culture medium* (the intracellular signal is neglectable because the intracellular volume is minute compared to the volume of medium), the culture medium of 24 h cultures in microplate wells was replaced with 100 μL of treatment medium containing studied compounds in various concentrations. After 1 h of treatment, oxidation was stimulated with 600 μM AAPH (10 μL of 6.6 mM solution in Hank's balanced salt solution (HBSS buffer)) for 30 min at 37 °C. Finally, 10 μL of 120 μM DCFH-DA was added by an automatic microplate reader dispenser to reach the final concentration of 50 μM, and kinetic monitoring of fluorescence was started. This method is a modification of the assay used originally for cells cultured in suspension.[20,27]

The method for measuring *intracellular antioxidant activity* is also described elsewhere[26,28] and has been shown to measure in particular intracellular activity.[29] After cells had been cultured for 24 h, medium was removed, and cells were washed twice with prewarmed PBS and then treated for 1 h with studied compounds in various concentrations and 50 μM DCFH in 100 μL of treatment medium. Subsequently, the treatment medium was removed, and wells were washed twice with PBS to remove extracellular fluorescent dye and sample residues. Then, 80 μL of prewarmed HBSS was added to sample wells and 100 μL to control wells used to measure unspecific fluorescence. The plate was preincubated in a microplate reader at 37 °C for 10 min, and 20 μL of 3 mM AAPH was dispensed into each sample well through an automatic dispenser of the plate reader.

An improved assay modification measuring the *effect of (mainly) extracellular antioxidant on intracellular oxidation* was developed and examined during the study. After 24 h culturing of the cells, the growth

medium was replaced by 80 μL of the treatment medium containing 50 μM DCFH-DA. After 1 h of incubation, wells were washed three times with prewarmed PBS, and studied compounds in various concentrations in the HBSS were pipetted into the wells (80 μL/well, except 100 μL/well for the controls of unspecific fluorescence). As described in the method for intracellular antioxidant activity assay above, the plate was preincubated in the microplate reader at 37 °C for 10 min and the oxidant AAPH (20 μL of 3 mM solution) was automatically dispensed to the wells prior to fluorescence monitoring.

The quantification of oxidation was done by calculating the AUC from t = 0 to t = 60 min. The net oxidation was obtained by subtracting the blank AUC from AUC of each sample. When the antioxidant assay was done in cell culture medium, the blank was the background sample without antioxidant and oxidant but containing DCFH-DA. When the intracellular oxidation was monitored, either the control sample without cells or the lowest fluorescence value of each individual sample, if lower than the fluorescence of the control sample, was used as a blank.

Copper-Initiated Pro-oxidant Activity (CIPA) Assay. The measurement of copper-initiated pro-oxidant activity of ellagitannin metabolites was modified from that of Cao et al.[30] to closely follow the protocol described above for the ORAC assay. In the assay, DCFH-DA acted as a fluorescent probe and Cu^{2+} (as $CuSO_4$) was used as a transition metal oxidant. Briefly, blanks and serial dilutions of urolithins A and B and ellagic acid (16−128 μM) were prepared in phosphate buffer (75 mM, pH 7.4, no EDTA) and pipetted (25 μL) to clear 96-well microplate wells. The final DMSO content was 1.25% and equal in all samples. One hundred and fifty microliters of 66.7 μM DCFH-DA was added to the wells, and the plate was incubated for 15 min at 37 °C before automatic dispension of 25 μL of 400 μM copper sulfate. After copper sulfate addition, the plate was mixed (5 s, 60 spm), and fluorescence emission was measured at 525 nm with excitation at 490 nm every 2 min for 90 min. The AUC values from t = 0 to t = 80 min were calculated, and the copper-initiated pro-oxidant activity was obtained by subtracting AUC determined in the presence of urolithin or ellagic acid but without copper from that of determined in the presence of both polyphenol and copper. The copper-initiated pro-oxidant activity induced by copper without polyphenol was set to 1, and values of other samples were related to it: pro-oxidant activity = $(AUC_{[polyphenol+copper]} - AUC_{[polyphenol]})/(AUC_{[copper]} - AUC_{[buffer]})$. The oxidation induced by polyphenols without copper was calculated by setting the oxidation in a buffer control to 1 and relating values of other samples to it.

Cyclic Voltammetry. Voltammetric measurements were performed using a PalmSens potentiostat (PalmSens, Utrecht, The Netherlands) equipped with PSTrace software (version 2.5.2). Screen-printed electrodes, consisting of graphite working (diameter of 3 mm) and counter electrodes and a Ag/AgCl reference electrode, were obtained from EcoBioService and Research (Florence, Italy). All electrodes showed small oxidation peak at potential +0.05 V originating from silver in the electrodes. However, it was confirmed using a small number of electrodes by two other manufacturers that this peak does not affect CV signals of the studied compounds. Prior to each electrochemical measurement, the electrodes were scanned in KNO_3−PBS solution, pH 7.4, for four cycles with a scan rate of 100 mV s^{-1} in the potential range from −0.1 to +1.0 V. The urolithins and ellagic acid were diluted to a concentration of 50 μM in PBS with the supporting electrolyte 0.1 M KNO_3. Electrochemical measurements were performed in a sample volume of 50 μL directly applied on the surface of the screen-printed electrode. The cyclic voltammograms were recorded at scan rates of 25, 50, 100, 200, and 400 mV s^{-1} in the potential range from −0.1 to +1.0 V. The scans were repeated 4−10 times in sequence to monitor the passivation of the electrode. The first cycle of each scan was used for determination of the oxidation potential and evaluation of the current response of studied compound.

Cell Proliferation Assay. The assay was performed using Cell Proliferation Kit XTT (Applichem, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, triturated HepG2 cells were seeded into 96-well plates at a density of 1×10^4 cells/100 μL/well. Twenty-four hours after seeding, the growth medium was removed and cells were washed twice with 150 μL of prewarmed PBS. The cells were treated in sextuplicate wells with each studied compound and concentration (10, 20, and 40 μM), respectively, for 24 h. For this, the compounds were diluted from stock solutions to a treatment medium consisting of growth medium and 10 mM HEPES buffer. The dilutions were prepared so that the concentration of DMSO was equal in all cultures (1.14%). After the treatment, 50 μL of cell proliferation assay reagent was added to each well, the plate was incubated for 60 min at 37 °C, and the absorbance was measured at 480 and 650 nm by a microplate reader. If the lowest absorbance values of samples had not reached the linear region of increase, the incubation was continued for 30 or 60 min. The relative proliferation was calculated by at first subtracting A_{650} from A_{480}, then subtracting A_{blank} from A_{sample}, and finally comparing the absorbance of the sample to that of a control not treated with the studied compound. The proliferation in untreated control sample was set to 100.

Statistical Analysis. Statistical significance of observed differences was assessed using a two-tailed Student's t test.

RESULTS AND DISCUSSION

Oxygen Radical Absorbance Capacity (ORAC) Assay. To confirm the previously reported antioxidant activity of ellagitannin metabolites,[15] we determined hydrophilic ORAC values for ellagic acid and urolithins A and B. The values were 4.25, 6.67, and 5.77 Trolox equiv, respectively, and the $ORAC_{uroA}/ORAC_{ellagic\ acid}$ ratio was 1.57 (Table 1). As a comparison, we obtained an approximately 2-fold higher (11.20 Trolox equiv) ORAC value for quercetin, one of the most studied strong antioxidants.[31] In the earlier study, urolithin B was not assayed at all, whereas ellagic acid and urolithin A were shown to be strong antioxidants with an $ORAC_{uroA}/ORAC_{ellagic\ acid}$ ratio of ∼2.0.[15] The ratio between the ORAC values was similar in both studies, but the ORAC values cannot be directly compared because we presented our results as Trolox equivalents, whereas the others reported raw data. In addition, the absolute ORAC values may vary depending on assay conditions and setup.[22]

Table 1. Antioxidant Activity of Ellagitannin Metabolites in ORAC Assay

analyzed compd	$ORAC_{ROO^\bullet}$ value[a] (Trolox equiv)
urolithin A	6.67 ± 0.11
urolithin B	5.77 ± 0.37
ellagic acid	4.35 ± 0.59
quercetin	11.20 ± 1.27

[a]Values are the average ± standard deviation of three independent assays done in sextuplicate.

Phenolic compounds (ArOH) have been shown to scavenge efficiently alkylperoxyl ($ROO^\bullet$) radicals,[32] and on the basis of electron density functional theory calculations, this is due to their ability to donate hydrogen from their phenolic hydroxyl group.[23]

$$ROO^\bullet + ArOH \rightarrow ROOH + ArO^\bullet \quad (1)$$

The HAT mechanism is dominant if the bond dissociation enthalpy between hydrogen and oxygen atoms in the phenolic hydroxyl group is relatively low (∼10 kcal/mol lower compared to unsubstituted phenol), whereas low ionization energy favors the SET mechanism (<45 kcal/mol compared to unsubstituted phenol).[23] To be an effective scavenger, a phenol compound must be also relatively stable as a free radical to react slowly with the substrate RH but rapidly with $ROO^\bullet$.

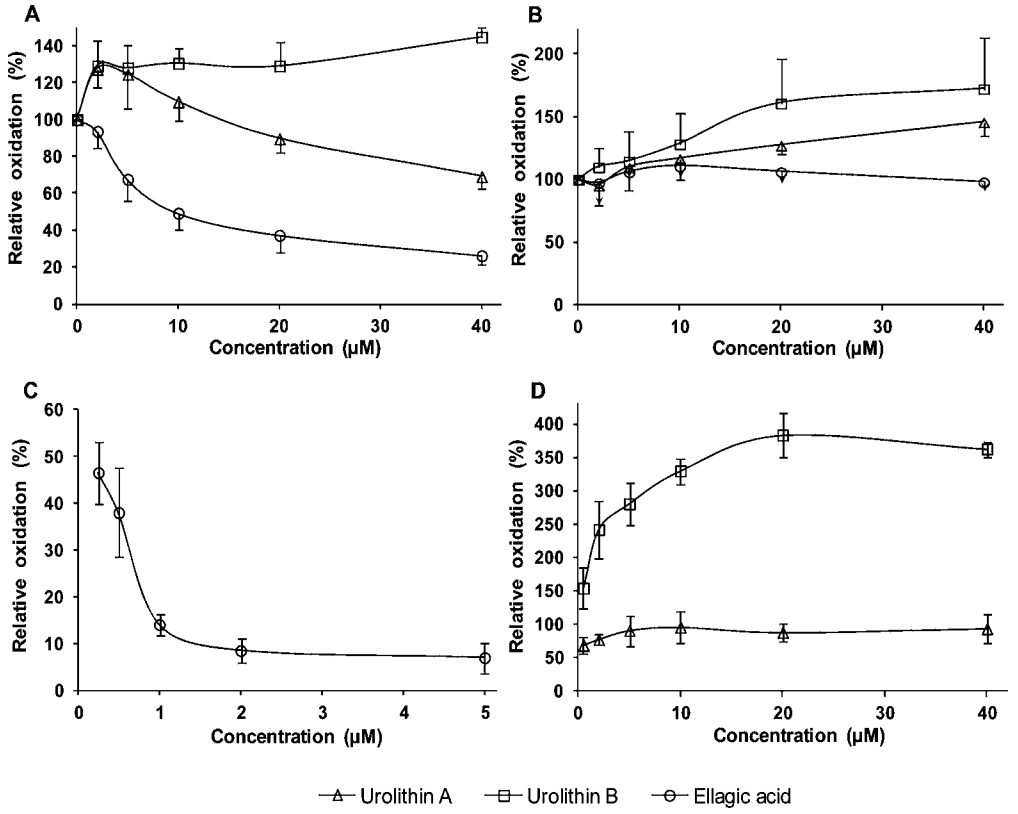

Figure 2. Cell-based antioxidant activity assays of ellagitannin metabolites. DCFH-DA was added to HepG2 cell cultures and oxidized by AAPH either in the cell cultures (A) or intracellularly in the HepG2 cells after extracellular DCFH-DA was removed by washing (B–D). In panel A, the indicated concentration of the polyphenol compound was present in culture medium during loading of the dye and monitoring of oxidation, whereas in panel B, the extracellular polyphenol was washed away together with the dye before oxidation was monitored. In panels C and D, cells were first loaded with the dye, and after extracellular dye was washed away, cells were incubated with the polyphenol and the oxidant. The presented data are averages of three to five independent experiments.

The greater antioxidant capacity of urolithins compared to ellagic acid may be due to the lower bond dissociation enthalpy of the phenolic hydrogen favoring the HAT mechanism and/or higher stability of the phenoxy radicals formed after donation of a hydrogen atom. The structure of urolithins enables conjugative resonance stabilization of the formed radicals, whereas ellagic acid has two electron-withdrawing groups in meta positions close to hydroxyl groups. The electron-withdrawing groups in meta positions tend to decrease the ability to donate phenolic hydrogen.[22,23] An increasing number of phenolic hydroxyl groups typically increases the antioxidant activity of a compound,[30] which was observed also in this study when the ORAC values of urolithins were compared. Although the antioxidant activity of urolithins appears to be mediated exclusively by the HAT mechanism, this does not mean that they would be less significant antioxidants. Actually, the HAT mechanism and oxidation induced by peroxyl radicals are considered to be biologically more relevant than the SET mechanism or oxidation induced by other oxygen radicals.[21]

Cell-Based Antioxidant Activity Studies. Several methods have been developed for measuring antioxidant activity in cell culture environment. We were particularly interested in intracellular oxidation reactions and wanted to use a method that is able to detect the HAT mechanism and uses peroxyl radicals to oxidize reporter dye. Urolithin A and ellagic acid have been shown to be antioxidants in a suspension culture of promyelocytic cell line HL-60 by a method where cells are pretreated with phorbol myristate acetate (PMA) oxidant and the studied compound.[20] The DCFH-DA dye was not added until the beginning of fluorescence monitoring. We opted for this method, although it measures mainly extracellular oxidation in the medium, but modified it so that AAPH was used as an oxidant instead of PMA. For assessing intracellular oxidation, we selected a method in which cells are first incubated with the studied compound and DCFH-DA and are then washed before AAPH oxidant is added in HBSS buffer.[26,28,29] During monitoring of fluorescence, the culture contains only intracellular DCFH that has become trapped in the cells.[26]

The results of the experiments are shown in Figure 2. *In cell culture medium*, urolithin A acted as an antioxidant, whereas urolithin B did not show any obvious concentration-dependent antioxidant or pro-oxidant activity (Figure 2A). Of interest, when the oxidation of DCFH-DA was monitored in complete medium without cells, fluorescence signals were markedly higher than in the measurements with cells (results not shown). Therefore, HepG2 cells are able to produce antioxidant molecules or excrete proteins that scavenge peroxyl radicals in the culture medium.

When the *intracellular antioxidant activity* was assessed, urolithins A and B showed no antioxidant properties; instead, they appeared as pro-oxidants (Figure 2B). Forty micromolar urolithin A increased oxidation (1.47 ± 0.12)-fold ($p < 0.01$) and 40 μM urolithin B (1.72 ± 0.41)-fold ($p < 0.01$). Ellagic acid was a strong antioxidant in cell culture medium (Figure 2A), but did not show any intracellular activity (Figure 2B), apparently due to its poor permeability through the plasma

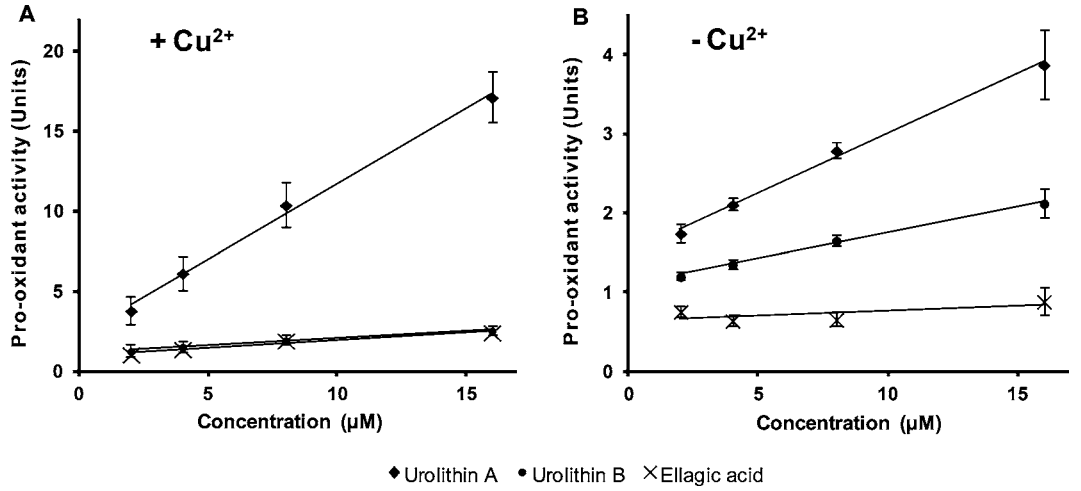

Figure 3. Copper-initiated pro-oxidant activity assay of ellagitannin metabolites. Polyphenols were incubated either in the presence (A) or in the absence (B) of 50 μM Cu^{2+}.

Table 2. Pro-oxidant Activity of Ellagitannin Metabolites in the CIPA Assay

		k^a	b^a	R^2	fold increase from 2 to 16 μM polyphenol[b]	average fold[c] increase induced by Cu^{2+}
urolithin A	+ Cu^{2+}	0.941	2.316	0.995	4.49	3.31 ± 0.97
	− Cu^{2+}	0.151	1.500	0.995	2.22	
urolithin B	+ Cu^{2+}	0.090	1.165	0.989	2.01	1.15 ± 0.06
	− Cu^{2+}	0.065	1.097	0.996	1.76	
ellagic acid	+ Cu^{2+}	0.095	1.004	0.953	2.28	2.31 ± 0.69
	− Cu^{2+}	0.013	0.648	0.477	1.17	

[a] k = slope and b = interception with y axis of curves presented in Figure 3. [b] Values are averages of three to four independent experiments [c] Fold increases induced by Cu^{2+} were calculated in all ellagitannin metabolite concentrations and an average of them was taken.

membrane[13,33] and the removal of extracellular ellagic acid by washing prior to fluorescence monitoring. These results agree well with previous work on HL-60 cells[20] reporting ellagic acid as a markedly stronger antioxidant compared to urolithins A and B showing no antioxidant activity.

We were not completely satisfied with the employed intracellular antioxidant activity assay because there were only traces of polyphenol left in the medium when fluorescence was measured and the assay required that the studied compound permeates into the cells for detection of any antioxidant activity. Thus, we modified the method to assay *the effect of extracellular antioxidants on intracellular oxidation*. To this end, cells were loaded first with DCFH-DA, and then the extracellular dye was washed away and cells were treated simultaneously with the AAPH oxidant and the studied compound in HBSS buffer. The loading time of 60 min saturates cells completely with the dye.[26] In this modification, peroxyl radicals are scavenged mainly extracellularly, and the oxidation of DCFH indicates the effect on intracellular oxidation reactions. In the assay, ellagic acid was a strong antioxidant having an EC_{50} value of <0.25 μM (Figure 2C). Surprisingly, urolithin A did not have a marked effect on either direction, whereas now urolithin B was a very strong pro-oxidant (Figure 2D). Actually, the oxidation reaction with urolithin B proceeded so rapidly that although the measurement time selected for quantitation of oxidation was shortened from 60 to 40 min, the reaction rate of oxidation was strictly constant during quantitation (fluorescence increased linearly) only when urolithin B concentration was ≤10 μM (Supporting Information Supplementary Figure 1). Therefore, the oxidation presented in Figure 1D for urolithin B concentrations of 20 and 40 μM is an underestimation of real values. In this assay, the studied compounds were in HBSS buffer during incubation with cells, and therefore serum proteins did not affect free radicals; neither did the cells have time after washing to excrete marked amounts of proteins to the HBSS nor were there present any metal ions from the cell culture medium.

Copper-Initiated Pro-oxidant Activity (CIPA) Assay. Results from cell-based experiments stimulated further studies on pro-oxidant activity of ellagitannin metabolites. The tendency of polyphenols to act as pro-oxidants is elevated in the presence of transition metal ions, such as Cu^{2+} or Fe^{2+}. One possible mechanism for this is that Cu^{2+} is first reduced to Cu^{+} by a polyphenol and subsequently reoxidized in a Fenton-like reaction with H_2O_2 or O_2, leading to the production of oxygen radicals.[34,35] In living cells, a small amount of hydrogen peroxide is produced as a result of cellular metabolism, and there are also transition metal ions present.[36]

We assayed the copper-initiated pro-oxidant activity of ellagitannin metabolites by a method that was modified from the original one[30] to follow more closely the ORAC assay protocol: 75 mM phosphate buffer (pH 7.4) was used in both the ORAC and CIPA assays, but AAPH was replaced with a transition metal oxidant copper, and consequently EDTA was excluded from the buffer in the CIPA assay. Both urolithins A and B were pro-oxidants in the CIPA assay even without copper induction (Figure 3B; Table 2). Urolithin A was a stronger pro-oxidant; similarly, it was a stronger antioxidant in

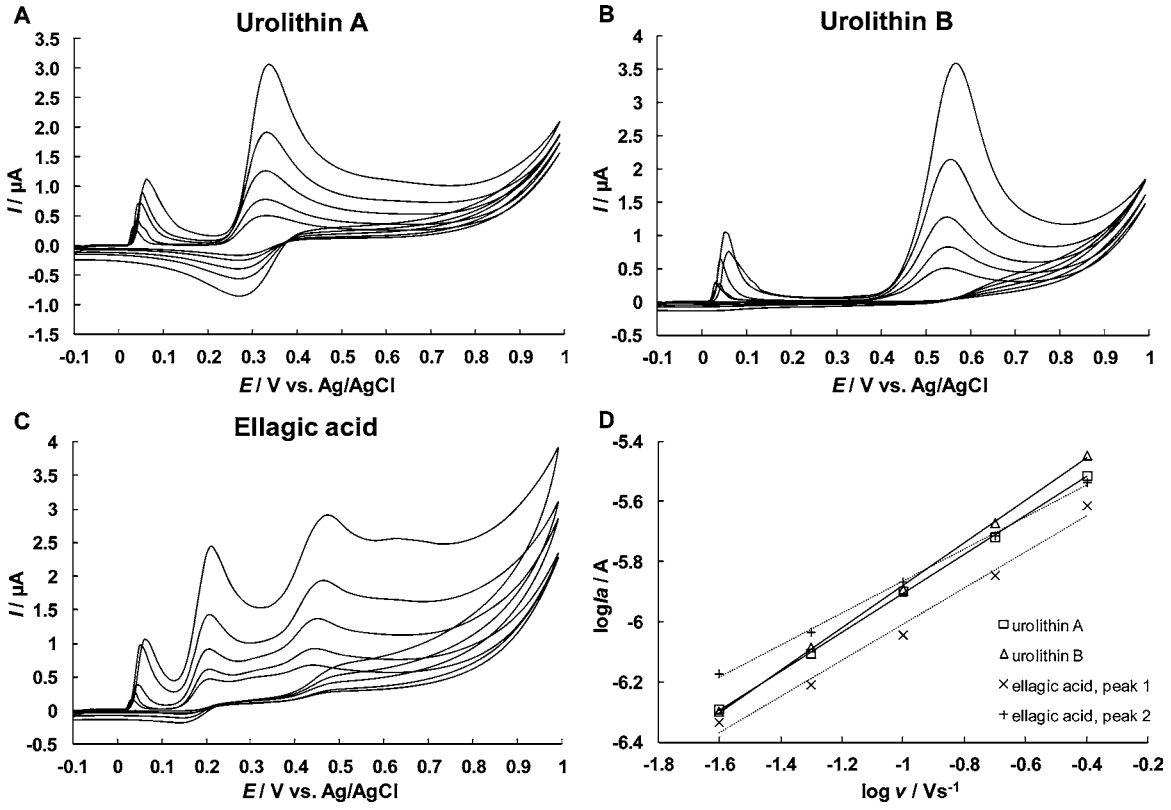

Figure 4. Cyclic voltammograms of 50 μM urolithin A (A), urolithin B (B), and ellagic acid (C) solutions at scan rates of 25, 50, 100, 200, and 400 mV s^{-1}. The first peak at 0.05−0.06 V is generated by silver in electrodes and was identical in the CVs of buffer controls. (D) Plot of the logarithmic peak currents as a function of the logarithmic scan rate. Urolithin plots are presented as solid lines and ellagic acid plots (two peaks) as dotted lines.

the ORAC assay. Ellagic acid showed antioxidant properties in concentrations from 2 to 8 μM, but at a concentration of 16 μM it appeared to be a weak pro-oxidant.

The copper induction increased the pro-oxidant activity of ellagitannin metabolites (Figure 3A; Table 2). It is important to note that all activity values presented are relative, and the values obtained with copper compare increases induced by copper, not total oxidation values. Urolithin A showed a distinct pro-oxidant behavior compared to ellagic acid and urolithin B. The copper-induced DCFH-DA oxidation in all urolithin A concentrations was on average 3.31-fold higher compared to the oxidation induced by copper in the buffer control. Without copper, the increase of urolithin A concentration from 2 to 16 μM increased DCFH-DA oxidation 2.21-fold, whereas with copper the induction was 4.48-fold. Therefore, urolithin A and copper ions interacted, enhancing the oxidation capacity of each other.

The addition of copper to urolithin B-containing samples and to a buffer control resulted in a nearly equal increase in DCFH-DA oxidation, because the average increase induced by copper was only 1.14-fold higher in the urolithin B samples than in the buffer control. Moreover, when the urolithin B concentration increased from 2 to 16 μM, the increases in the pro-oxidant activity with and without copper were very similar, 2.01- and 1.76-fold, respectively. These results demonstrate that urolithin B and copper ions do not interact but increase the oxidation of DCFH-DA independently of each other. They both induced significant increases in oxidation as the addition of copper to the buffer control resulted in a 1.74-fold increase in absolute oxidation (results not shown).

Also, ellagic acid interacted with copper ions, but the effect on pro-oxidant activity was weaker compared to the effect of urolithin A and copper interaction. Without copper, ellagic acid was a weak pro-oxidant only in high concentrations, whereas the induction with copper resulted in an average 2.31-fold increase in DCFH-DA oxidation when compared to the induction in buffer control. When the ellagic acid concentration increased from 2 to 16 μM, the oxidation of DCFH-DA increased only 1.17-fold without copper but 2.28-fold with copper.

Urolithin B with its one hydroxyl group is not able to form more than one bond with copper ion, whereas urolithin A and ellagic acid have two and four hydroxyl groups, respectively, enabling interactions with two or four metal ions or alternatively more than one interaction with one ion. The polyphenols containing one hydroxyl group have been observed to be poor pro-oxidants also earlier when stimulated by metal ions.[30,34] Another determining factor could be differences in redox properties of urolithins A and B, because polyphenols are themselves initially oxidized by the metal ions when they act as pro-oxidants and then may undergo redox cycling.[34,35,37]

Electrochemical Oxidation of Ellagitannin Metabolites. The electrochemical oxidation of ellagic acid has been studied earlier,[38−40] but that of the urolithins is completely unknown. We were especially interested in investigating whether the difference of one hydroxyl group between urolithins A and B has a profound effect on their electrochemical oxidation because their behavior differed markedly in the CIPA assay. In addition, electrochemistry provides a novel approach and perspective for observation of the redox properties of the ellagitannin metabolites.

The electrochemical oxidation of ellagitannin metabolites in aqueous buffer (PBS containing 0.1 M potassium nitrate, pH 7.4) was investigated by cyclic voltammetry (CV) employing screen-printed carbon electrodes. The cyclic voltammograms are presented in Figure 4. The CV data of urolithin A (Figure 4A) showed both anodic and cathodic peaks at each scan rate. The peak potential separation (ΔE_p) was approximately 60 mV, agreeing with the theoretical value (59 mV) for a reversible one-electron process.[41] However, the anodic peak currents were typically twice as high as the cathodic peak currents ($I_{pa}/I_{pc} \sim 2$), suggesting a quasireversible redox behavior in which the oxidized product reacts to another compound (EC mechanism), but with a reaction rate slow enough for some of the oxidized product to be reduced back to the original compound in reverse scan.[41] Because ΔE_p referred to the one-electron process, the phenoxy radicals of urolithin A were reduced back to urolithin A in the reverse reaction, not the phenoxonium ions (Figure 1B). In addition, repeated scanning led to a decrease in current and an increase in oxidation potential, indicating passivation of electrode and further supporting the EC mechanism. The cyclic voltammogram of urolithin B revealed an irreversible redox reaction as no cathodic peak was observed (Figure 4B). Also, this reaction likely followed the EC mechanism, but the reaction kinetics differed as compared to urolithin A oxidation, the oxidized urolithin B reacting rapidly further. The passivation of the electrode was more vigorous than with urolithin A or ellagic acid, suggesting formation of a more unstable phenoxy radical that reacted into products interfering with the electrode surface as observed for other polyphenols as well.[42] The oxidation potential of urolithin B was also higher than that of urolithin A, 0.56 and 0.33 V, respectively (Table 3), supporting the assumption that the bond between phenolic oxygen and hydrogen could have lower dissociation enthalpy in urolithin A due to another phenolic hydroxyl contributing to charge distribution in aromatic rings.

Table 3. Electrochemical Oxidation Potentials (E_{pa}) of Ellagitannin Metabolites in Cyclic Voltammetry and the Slope (k) and R^2 Values of Linear Regression of the Plot of Logarithmic Peak Currents versus Logarithmic Scan Rate (Figure 4D)

	E_{pa}[a] (V)	k	R^2
urolithin A	0.332 ± 0.005	0.64	0.9996
urolithin B	0.556 ± 0.009	0.70	0.9993
ellagic acid, peak 1	0.210 ± 0.000	0.60	0.9867
ellagic acid, peak 2	0.452 ± 0.013	0.53	0.9986

[a]All values presented are averages of at least three independent experiments; all five scan rates were used to determine E_{pa}.

The irreversible oxidation of urolithin B may imply that its ability to undergo redox cycling differs from the cycling capacity of urolithin A, and this may partly explain why urolithin B showed weaker pro-oxidant activity in the CIPA assay. Furthermore, the phenoxy radical formed from urolithin B is likely to be reactive and unstable. Hence, it would react rapidly further, for example, in culture medium with serum proteins or by polymerizing. In the HBSS buffer it does not have macromolecules or other radicals to react with in addition to cells, DCFH dye, and other urolithin B molecules. This may explain why urolithin B was an extremely strong pro-oxidant when it was applied onto cells in the HBSS buffer and its effect on intracellular oxidation was monitored (Figure 2D).

The CV data for ellagic acid showed two clearly distinct oxidation peaks (E_{pa} = 0.21 V and E_{pa} = 0.45 V) at neutral buffered solution, whereas the cathodic peaks were remarkably small, suggesting a quasireversible reaction (Figure 4C). The results are in agreement with previously reported electrochemical studies of ellagic acid.[38−40] The first oxidation peak is attributed to the formation of ellagic acid phenoxy radical by the release of one electron and proton. The second oxidation peak forms when the phenoxy radical is oxidized to a quinone or a semiquinone form that may undergo further dimerization or polycondensation reactions producing compounds that can also be oxidized. This explains the nearly irreversible nature of ellagic acid oxidation and may explain the third peak that is observed in alkaline conditions in the CV analysis of ellagic acid[39] and was very weakly detected in our study at pH 7.4 with the highest scan rate of 400 mV s^{-1} at a potential of 0.65 V.

The influence of the scan rate on the oxidation of ellagitannin metabolites was analyzed at scan rates of 25, 50, 100, 200, and 400 mV s^{-1}. Linear relationships of the logarithmic peak currents and the logarithmic scan rates were observed in all studied ellagitannin metabolites (Table 3; Figure 4D). The slopes of linear regression point to mixed adsorption−diffusion-controlled processes as they lay between 0.5 (theoretical value for diffusion-controlled process) and 1 (theoretical value for adsorption-controlled process). Higher concentrations of ellagitannin metabolites (0.5 and 1 mM) were also tested. However, the peak shape was broadened, and the passivation of electrodes was rapid (results not shown).

Effect of Ellagitannin Metabolites on Cell Proliferation and Metabolic Activity. The discovered pro-oxidant activities of urolithins both inside the cells and in cell culture promote oxidative stress and presumably affect also cell growth and cellular metabolism. To assess this, we employed an assay that directly measures cellular metabolic activity by monitoring XTT reduction to formazan by the mitochondrial respiratory chain.[43] Under defined conditions, it reflects the number of viable cells and thus cell proliferation between selected time points. We observed that 48 h of incubation with 40 μM ellagic acid did not induce any changes in cell proliferation, whereas incubation with urolithin A or B led to a marked decrease in proliferation (Figure 5). Forty micromolar urolithin A decreased the growth of HepG2 cells to 51.3% ($p < 0.001$) and 40 μM urolithin B, to 73.0% ($p < 0.01$), compared to a control treated with DMSO vehicle. Considering that urolithins are able to pass cell membrane and increase intracellular oxidation, whereas ellagic acid is not cell membrane permeable and did not induce any intracellular response (Figure 2B),[13,33] the proliferation-decreasing effect of urolithins is likely dependent on their intracellular localization and ellagic acid did not affect proliferation because it cannot enter into the cells.

When a similar assay was done in the DMEM growth medium without cells, urolithins did not have any effect on XTT reduction (results not shown). Therefore, all changes observed in the cell proliferation assays resulted from cell metabolism. Urolithin A with its two functional groups had a slightly stronger effect than urolithin B. In earlier studies, others have stated that urolithins do not affect cell growth.[17,20] However, HepG2 is a hepatocellular carcinoma cell line that has maintained a number of specialized functions and enzymatic systems of liver cells that may result in an ability to undergo more complicated metabolic processes and to react

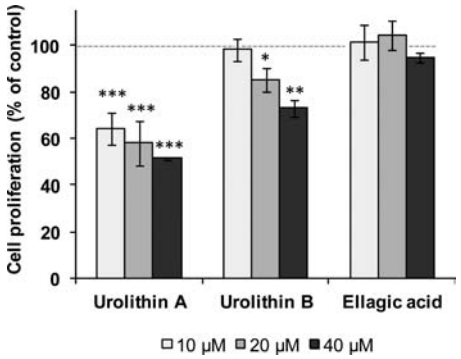

Figure 5. Cell proliferation assay of ellagitannin metabolites. Values are averages ± standard deviation of three independent determinations done in sextuplicate samples. The value of samples treated with only DMSO vehicle was set to 100%. In addition, untreated cell samples incubated without phenolics and DMSO were prepared and showed values of 105.7 ± 8.5% in the assay. ***, **, and * correspond to $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively.

more sensitively to changes in environment or to compounds added.[44] Recently, urolithins were reported to inhibit also proliferation of human bladder cancer T24 cells.[45]

We also examined how a short incubation with urolithins affects the metabolic activity of HepG2 cells (with short incubation times metabolic activity is a more suitable term than cell proliferation). The aim was to follow assay conditions used in the cell-based antioxidant assays as closely as possible. No oxidant was added onto cells, and instead of DCFH-DA, only DMSO vehicle was added. When the assay protocol followed the antioxidant activity assay in cell culture medium, the activation reagent of the cell proliferation assay was added immediately after the DMSO vehicle to mark the addition of fluorescent dye in the antioxidant activity assay. After 1 h of culture with 40 μM urolithin A, the metabolic activity was only 67.5 ± 6.3% and that with urolithin B 64.8 ± 10.5% compared to the control ($n = 3$).

When the assay followed the improved method for monitoring the effect of extracellularly applied antioxidant on intracellular oxidation, the activation reagent was added together with urolithins in HBSS buffer. After 1 h of incubation with 40 μM urolithin A or B, the metabolic activity was decreased to 68.0 ± 5.4 and 71.6 ± 1.2%, respectively ($n = 3$), as compared to the metabolic activity of the control. Forty micromolar ellagic acid did not have any effect on metabolic activity in either of the assay conditions (94.3 ± 4.9 and 100.7 ± 4.0%, respectively, $n = 3$), indicating that the decrease in metabolic activity in urolithin-containing cultures was caused by the metabolites and not by a change of a new medium or a short stay outside the cell incubator. Therefore, even a short incubation with urolithins had a relatively similar effect on the metabolic activity of HepG2 cells as incubation for 48 h had, suggesting that the cytostatic effect cannot be only a consequence of the oxidation of urolithins in the culture medium. Because the decreased metabolic activity directly affects also oxidation reactions in cells, the detected increases in the intracellular oxidation in cell-based assays actually underestimate the real pro-oxidant activities of urolithins.

Effect of Storage on Urolithin A. Urolithin A was synthesized, and the first stock solutions for antimicrobial studies (results unpublished) were prepared nearly 1 year before we started antioxidant activity assays. In the cell-based studies, originally by chance, also an old stock solution was assayed. In cell culture medium, the urolithin A of old stock solution showed pro-oxidant behavior, although the urolithin from new stock solutions prepared from the same synthesis lot but solubilized later acted as an antioxidant (Supporting Information Supplementary Figure 2). The urolithin A from old stock solutions appeared as a stronger pro-oxidant also in the intracellular assay, but the difference was not statistically significant. The differences between old and new stock solutions were confirmed using several fresh or new (<3 months from preparation) solutions and two older (>1 year from preparation) stock solutions. The old stock solutions were stored at −20 °C after preparation, but the new stock solutions were stored mostly at −80 °C. The ORAC values of old stock solutions were >90% of the values of new stock solutions (data not shown).

The HPLC-DAD/MSD analysis revealed that a very small fraction of urolithin A dimerized during storage in solutions (Supporting Information Supplementary Figures 3 and 4). A m/z of 453.1 indicates that the dimer was a result of oxidative loss of two electrons and protons. The similarity of the UV absorbance spectrum of the supposed dimer to the spectrum of monomeric urolithin confirmed the identification (Supporting Information Supplementary Figure 5). The presence of dimerized urolithin A in the old stock solution may aid in shifting the redox balance to a more oxidative direction, which was observed in cell-based studies. The dimer has four oxygen atoms able to form interactions, or the dimer could have a capability to break down into radicals that then become reduced.

Conclusion and Future Aspects. This study assessed the redox properties of ellagic acid and its intestinal metabolites, urolithins, by a number of methods demonstrating how strongly their antioxidant and pro-oxidant activities depend on the assay system and conditions. The results illustrate how important it is to study antioxidant and pro-oxidant capacities in parallel and how informative the cyclic voltammetry technique is also when biological redox reactions are investigated. In addition, two method modifications were proposed: one for assaying the effects of extracellular antioxidants on intracellular redox reactions and another for assaying pro-oxidant activity in conditions similar to ORAC assay conditions. To our knowledge, this is the first study investigating the pro-oxidant activity and electrochemical oxidation of urolithins.

Currently, antioxidant activity assays are typically done using chemical in vitro assays and in conditions making the assay as sensitive as possible, that is, favoring antioxidant activity. Assaying pro-oxidant activity is usually neglected. In this study, the recently much criticized[46] ORAC assay presented ellagitannin metabolites as relatively strong antioxidants, whereas the CIPA assay done in the same buffer as the ORAC assay but employing a more natural oxidant, transition metal ion Cu^{2+}, instead of AAPH revealed their pro-oxidant capacity. Cell culture media are said to favor pro-oxidant activity.[47] However, transition metal ions together with small amounts of hydrogen peroxide are present also in tissues and cells. Furthermore, we observed that the cells significantly decreased oxidation in the cultures compared to the oxidation in the medium controls without cells. This raises a question: whether the cell culture environment might indeed provide a more natural environment for antioxidant activity assays than the chemical in vitro assays that lack all other antioxidants and natural defensive mechanisms of cells and tissues. In the future,

more physiologically relevant assay conditions could be reached, for example, by establishing a standardized biological fluid (natural or artificial) to replace or complement buffers in the assay systems or by developing oxidizable probes so that a probe or rather a set of probes could be added directly to a serum sample or cell culture.

Finally, this study draws attention to several major challenges facing scientists in the field of antioxidant studies: (a) standardized and physiologically more relevant assays are required; (b) consequently, more collaboration between analytical chemists and medical scientists is needed; (c) redox properties of parent polyphenolic compounds and their intestinal metabolites may differ substantially; (d) therefore, redox properties of metabolites require systematic investigations; and (e) assay systems that can genuinely dissect extracellular and intracellular effects in the continuous presence of antioxidant should be developed and established.

■ ASSOCIATED CONTENT

Supporting Information

Five additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AAPH, 2,2′-azobis[2-methylpropionamidine] dihydrochloride; AUC, area under curve; CIPA, copper-initiated pro-oxidant activity; CV, cyclic voltammetry; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; HAT, hydrogen atom transfer; HBSS, Hank's balanced salt solution; ORAC, oxygen radical absorbance capacity; PBS, phosphate-buffered saline; SET, single electron transfer

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